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Research Article

Licochalcone E Present in Licorice Suppresses Lung Metastasis in the 4T1 Mammary Orthotopic Cancer Model

Soo Jin Kwon[1], So Young Park[3], Gyoo Taik Kwon[1], Ki Won Lee[3,4,*], Young-Hee Kang[1], Myung-Sook Choi[5], Jong Won Yun[6], Jae-Ho Jeon[2], Jong Gab Jun[2], and Jung Han Yoon Park[1,3,*]

Abstract

We investigated whether licochalcone E (LicE), a phenolic constituent of licorice, inhibits mammary tumor growth and metastasis using animal and cell culture models. 4T1 mammary carcinoma cells were injected into the mammary fat pads of syngeneic BALB/c mice. Starting 7 days after the injection, the mice received LicE (7 or 14 mg/kg body weight/day) via oral gavage for 25 days. LicE suppressed solid tumor growth and lung metastasis, but did not exhibit kidney or liver toxicity. In tumor tissues, LicE treatment induced a reduction in the expression of Ki67, cyclins, and cyclin-dependent kinases and stimulated apoptosis with increased expression of Bax and cleaved caspase-3 but decreased expression of Bcl-2. In addition, LicE decreased expression of CD31, vascular endothelial growth factor (VEGF)-A and C, VEGF-receptor 2, lymphatic vessel endothelial receptor-1, CD45, cyclooxygenase-2, inducible nitric oxide synthase, and hypoxia inducible factor-1α in tumor tissues. In lung tissues, LicE reduced the levels of proinflammatory cytokines and angiogenesis/metastasis-related proteins. In mammary cancer cell cultures, LicE (5–20 μmol/L) dose dependently inhibited cell migration and invasion. LicE inhibited secretion of matrix metalloproteinase-9, urokinase-type plasminogen activator and VEGF-A, and stimulated secretion of tissue inhibitor of metalloproteinase-2 in MDA-MB-231 cells. In addition, LicE inhibited tube formation of vascular endothelial cells. We show that LicE administration suppressed tumor growth and lung metastasis in the mouse model in conjunction with LicE inhibition of cell migration, invasion, and tube formation *in vitro*. Reduced tumor growth and metastasis in LicE-treated mice may be, at least in part, attributed to reduced inflammation and tumor angiogenesis. *Cancer Prev Res; 6(6); 603–13. ©2013 AACR.*

Introduction

Breast cancer is one of the most common malignant diseases and the most aggressive cancer type of women worldwide (1). Despite early detection and apparently complete surgical resection, many patients still die of metastatic cancer that remains undetected at diagnosis (2). Therefore, development of new strategies for preventing breast cancer metastasis is urgently needed. Metastasis is a complex process that includes the separation of cancer cells from their primary tumor site by breaking through the stromal tissue, transportation through lymphatics or blood vessels, adhesion to the basement membrane, and invasion at the target organ of distant metastasis [reviewed (3)]. Identification of dietary bioactive compounds lacking toxicity and capable of blocking more than one of these processes could be a good strategy to prevent metastasis.

Many seemingly healthy people can harbor microscopic tumors in an asymptomatic and undetectable state for several years. These tumors do not progress in the absence of inflammation or angiogenesis, which are 2 host-dependent and interdependent hallmarks of cancer [reviewed in (4)]. Chronic inflammation promotes angiogenesis (5) and inflammatory reactions play crucial functions at multiple phases of cancer development including initiation, promotion, malignant conversion, invasion, and metastasis [reviewed in (6)]. Therefore, dietary bioactive compounds that inhibit both inflammation and angiogenesis can be particularly useful as cancer chemopreventive agents.

Licorice has been used in food and medicinally for thousands of years. In Chinese medicine, licorice species including *Glycyrrhiza inflata* (*G. inflata*) remain one of the most prescribed herbs and contain unusual retrochalcones:

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licochalcone A to E and echinatin. Licochalcone A exhibits a variety of biologic properties including antiinflammatory and antitumorigenic activities (7, 8). Licochalcone E (LicE; Structure in Supplementary Fig. S1) has recently been isolated and characterized from the roots of *G. inflata* (9) and has been shown to possess antidiabetic effects (10) and cytotoxic effects against A549 lung cancer cells, SK-OV-3 ovarian cancer cells, SK-MEL-2 melanoma cancer cells, and HCT-15 colon cancer cells (11).

In this study, we attempted to evaluate whether LicE inhibits mammary cancer promotion using a BALB/c mouse orthotopic model in which the mice were injected with syngeneic 4T1 murine mammary carcinoma cells. It has been established that solid tumor growth and metastatic spread of 4T1 cells closely mimic stage IV breast cancer when 4T1 cells are injected into the mammary fat pads of BALB/c mice (12). In the present study, the oral administration of LicE significantly inhibited solid tumor growth and lung metastasis of mammary cancer cells. Also, LicE inhibited tumor angiogenesis and lymphangiogenesis, as well as inflammatory status in tumor and lung (target organ) tissues. *In vitro* cell culture studies showed that LicE directly inhibited the migration and invasion of both MDA-MB-231 human breast cancer cells and 4T1 cells.

Materials and Methods

Reagents

Antibodies against vascular endothelial growth factor (VEGF)-A and C, platelet endothelial cell adhesion molecule (PECAM)-1 (CD31), cyclin-dependent kinase (CDK)2, CDK4, cyclin A, cyclin D1, Bax, Bcl-2, and cleaved caspase-3 were purchased from Santa Cruz Biotechnology. Antibodies to Ki67, lymphatic vessel endothelial hyaluronan receptor-1 (LYVE-1), and hypoxia-inducible factor (HIF)-1α were purchased from Abcam. Antibodies against tissue inhibitor of metalloproteinase (TIMP)-2 and urokinase-type plasminogen activator (uPA) were purchased from R&D Systems. Antibodies to cyclooxygenase (COX)-2 and inducible nitric oxide synthase (iNOS) were purchased from BD Transduction Laboratories. We prepared LicE using a previously described method (13). In our previous work, the authenticity of this compound was verified by nuclear magnetic resonance (NMR) spectroscopy. The NMR spectra (^{1}H, ^{13}C) of this compound recorded on Varian Mercury-300 MHz Fourier-Transform-NMR (Varian Inc.) were completely matched to the literature values (13–15). The LicE preparation used in the present study was determined to be of 93.6% purity, using an Agilent 110 series HPLC system (Agilent Technologies).

Cell lines and culture conditions

MDA-MB 231 human breast cancer cells (#HTB-26) and 4T1 murine mammary carcinoma cells (#CRL-2539™), and SV40-transformed endothelial cells (SVEC4-10, #CRL-2181) were obtained from the American Type Culture Collection (ATCC) in May 2010. All experiments with these cells were conducted within 1 month (within 7 passages) of resuscitation of frozen cell stocks prepared within 3 passages of receipt from the ATCC. The ATCC ensures authenticity of these cell lines using short tandem repeat (STR) analyses. MDA-MB 231 cells were maintained in Dulbecco's modified Eagle's medium: Nutrient Mixture F12 (Ham; 1:1; DMEM/F12, Gibco™, Invitrogen) containing 100 mL/L of fetal bovine serum (FBS), 2 mmol/L of L-glutamine, and 2 mg/L of insulin. 4T1 cells and SVEC were cultured in DMEM containing 100 mL/L of FBS. All cell maintenance media contained 100,000 U/L of penicillin and 100 mg/L of streptomycin. Human umbilical vein endothelial cells (HUVEC, C-2517A) were used within 5 passages of receipt (February 2013; Lonza) and were cultured with an EGM-2 bulletkit (CC-3162, Lonza). Lonza ensures the quality of HUVEC.

Animals and study design

All animal experimental protocols were approved by the Animal Care and Use Committee of Hallym University (Protocol approval #: Hallym2011-05). Female, 4-week-old BALB/c mice were purchased from Orient Bio and were allowed to acclimate for 1 week before use in animal research facility of Hallym University. The mice were fed an AIN76A diet (Research Diets) and water *ad libitum*. After acclimatization, the mice were divided randomly into 4 groups: (i) control [sham-injected, corn oil (vehicle)-fed], (ii) 4T1 cell-injected + corn-oil fed, (iii) 4T1 cell-injected + 7 mg LicE/kg body weight/day, and (iv) 4T1 cell-injected + 14 mg LicE/kg body weight/day. 4T1 cells (5×10^{4} cells suspended in 0.1 mL matrigel; BD Biosciences) were injected into the inguinal mammary fat pads. The control group (i) received a sham-injection of 0.1 mL of matrigel only. One week after the 4T1 cell injection, the mice were subjected to oral gavage with vehicle or LicE (7 or 14 mg/kg body weight/day) for 25 days. LicE was diluted in corn oil for administration, and all animals received the same amounts of corn oil. The tumor volume was measured with a set of calipers and calculated as $0.52 \times$ long diameter $\times$ short diameter^{2} (16). Thirty-two days after the 4T1 cell injections, the animals were anesthetized via an intraperitoneal injection of 2.5% avertin (10–15 μL/g body weight) and blood was collected from the orbital venous plexus. After blood collection, all mice were sacrificed by carbon dioxide asphyxiation, and the tumors, lungs, livers, kidneys, and spleens were isolated from the mice and weighed. The right lungs were fixed in Bouin's solution and lung metastatic nodules were counted and the total tumor volumes were estimated as described previously (17, 18).

Sera were prepared to determine the concentrations of creatinine and activities of aspartate aminotransferase (AST) and alanine aminotransferase (ALT). The serum levels of creatinine were measured using a creatinine assay kit (BioVision). The activities of AST and ALT were estimated using assay kits (Thermo Fisher Scientific).

Protein array and enzyme-linked immunosorbent assay (ELISA)

Lung tissue lysates were prepared from the left lungs as described (19) and pooled (15 animals per group). The

differences in the levels of angiogenesis-related proteins and cytokines in the pooled samples *were assessed using a mouse angiogenesis array kit (Catalog # ARY015; R&D Systems) and mouse cytokine array kit, A (Catalog # ARY006; R&D Systems), respectively. The levels of monocyte chemotactic protein (MCP)-1, MMP-9, and interleukin 1 receptor antagonist (IL-1ra) of individual lung tissue lysate samples were estimated using ELISA kits (R&D Systems).

Immunohistochemical (IHC) and immunofluorescence (IF) staining

Five mice from each group were randomly selected for IHC and IF staining. Tumors were divided into 4, and the upper left quadrant was fixed in 4% paraformaldehyde for IHC analysis and the upper right quadrant was frozen for IF staining. The IHC and IF analyses were conducted as previously described (20). Apoptotic cells were identified via terminal deoxynucleotidyl transfer-mediated dUTP nick-end labeling (TUNEL) staining as previously described (21). Photographs were obtained using an AxioImager microscope (Carl Zeiss). The number of immunopositive cells and staining intensity was assessed in 5 random high-power fields (200×) per section (2 sections per mice × 5 mice per group) using the Automatic Measurement Program (Axiovision Rel 4.6 Program).

Transwell migration and invasion assays

For migration assay, 4T1 and MDA-MB-231 cells were subjected to 24 hours of serum deprivation in DMEM and DMEM/F12, respectively, supplemented with 10 mL/L charcoal-stripped FBS. They were then plated onto filters (8 μm-pore size) in transwell inserts (Corning Costar; Corning, NY) in 24-well plates at 2.5×10^4 cells/filter and treated with or without various concentrations of LicE. The undersides of membranes were precoated with 10 μg of type IV collagen. The lower chamber of each well was filled with DMEM (4T1 cells) or DMEM/F12 (MDA-MB-231 cells) containing 10 mL/L of charcoal-stripped FBS and 1 mL/L of bovine serum albumin. Cell migration was allowed to proceed for 12 hours (4T1 cells) or 4 hours (MDA-MB-231 cells). Cells were then removed from the upper surface of the membranes with a cotton swab, and cells that had migrated to the lower surface were stained with hematoxylin and eosin (H&E). Dried membranes were then cut out, and were mounted on glass slides in immersion oil. The numbers of migrated cells at least 8 random high-power fields from each of duplicated membranes were counted for each experimental condition, and were expressed as a percentage of the untreated control cells. For the invasion assay, identical procedures were conducted as those described in the migration assay except that cells were plated on a matrigel-coated transwell filter (BD Biosciences). Cell invasion was allowed to proceed for 18 hours (4T1 cells) or 8 hours (MDA-MB-231 cells). All migration and invasion assays were repeated at least 3 times. The viability of cells was estimated as previously described (22).

Tube formation assay

HUVEC (50,000 cells/well) and SVEC4-10 (75,000 cells/well) were seeded in endothelial cell basal medium-2 (Lonza) and DMEM, respectively, into 24-well plates which had been precoated with 0.3 mL of matrigel. Various concentrations of LicE were added into the wells, and the assay plates were incubated at 37°C for 2 hours. Tubular structures were photographed, and the total length of tube formation was measured by the Motic Images Advanced 3.2 system.

Western blot analysis and gelatin zymography

MDA-MB-231 cells (1×10^6 cells/100 mm culture dish) were plated in DMEM/F12 containing 100 mL/L of FBS. The cells were then serum-starved for 24 hours in DMEM/F12 and treated with or without various concentrations of LicE for 24 hours. Conditioned media were collected and concentrated, and the proteins in the concentrated conditioned media were subjected to Western blot analyses as previously described (23). The signal was detected with a chemiluminescence detection system. The relative abundance of each band (TIMP-2, uPA, and VEGF-A) was quantified using the Bio-profile Bio-ID application (Vilber-Lourmat) and the control (0 μmol/L LicE) levels were set to 100%.

To estimate matrix metalloproteinase (MMP) secretion, gelatin zymography was conducted using concentrated conditioned media as previously described (23). Conditioned media of HT1080 human fibrosarcoma cells which contain MMP-9 (24) and MMP-2 (25) were used as a positive control.

For all *in vitro* cell cultures for Western blotting and gelatin zymography as well as transwell migration, invasion and tube formation assays, LicE was dissolved in dimethyl sulfoxide (DMSO) and all cells were treated with DMSO at a final concentration of 0.01%.

Statistical analyses

The data were expressed as the means ± SEM and analyzed via analysis of variance. Differences between treatment groups were evaluated via Student t test (for the comparison between 2 groups) or Duncan's multiple range test (for the comparison among 3 to 4 groups), using SAS for Windows version 9.2 software (SAS Institute). Differences were considered significant at $P < 0.05$.
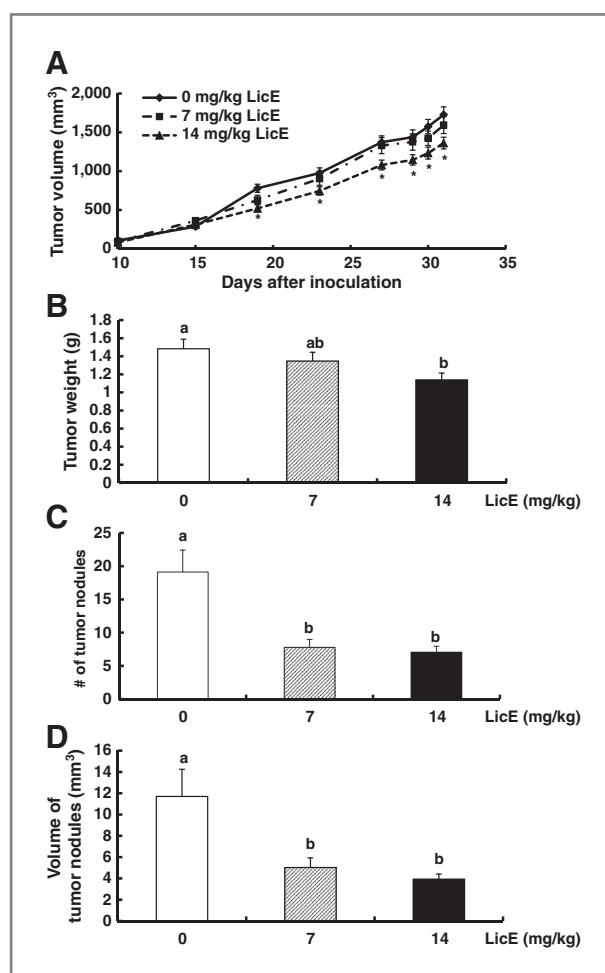
Results

Oral administration of LicE inhibits solid tumor growth and lung metastasis in BALB/c mice injected with 4T1 cells

To determine whether oral administration of LicE inhibits the solid tumor growth and metastasis of mammary cancer cells *in vivo*, we used female BALB/c mice in which 4T1 mammary carcinoma cells were injected into the mammary fat pads. The injected 4T1 cells typically formed a solid tumor in the mammary fat pad, which became visible 1 week after the injection, and the volume of tumors increased until the day of sacrifice. Starting one week after the injection of 4T1 cells until sacrifice, the mice were fed

LicE (7 or 14 mg/kg) via oral gavage for 25 days. LicE administration at 14 mg/kg significantly suppressed tumor growth, whereas tumor growth was not affected by 7 mg/kg LicE (Fig. 1A). At the end of the experiment, tumor weights were lower in the mice treated with 14 mg/kg of LicE as compared with mice treated with vehicle only, whereas tumor weights were not significantly different between mice receiving 7 mg/kg of LicE and vehicle (Fig. 1B).

The oral administration of LicE (7 or 14 mg/kg) for 25 days significantly reduced the number and volume of tumor nodules in the lung. The 2 doses of LicE were equally effective in reducing the number and volume of tumor

Figure 1. Oral administration of LicE inhibits solid tumor growth and lung metastasis in BALB/c mice injected with 4T1 cells. 4T1 cells (5×10^4 cells suspended in 0.1 mL Matrigel) were injected into the inguinal mammary fat pads of female BALB/c mice. One week after the injection, the mice were subjected to oral gavage with corn oil (vehicle) or LicE (7 or 14 mg/kg body weight/day) for 25 days. A, the tumor volume (mean ± SEM, $n = 15$) was measured using calipers and calculated using the formula (0.52 × long diameter × short diameter^2). *Significantly different from the vehicle-fed group, $P < 0.05$. B, 32 days after the injection, all mice were sacrificed, and the tumors were excised from the mice and weighed. C and D, lungs were fixed in Bouin's solution. The numbers (C) and volumes (D) of tumor nodules in the lung were determined. Each bar represents the mean ± SEM ($n = 15$). Means without a common letter differ, $P < 0.05$.

nodules in the lung (Fig. 1C and D). The body weight of the mice was not affected by 4T1 cell injection or LicE administration. The weights of liver, lung, and spleen were higher in mice injected with 4T1 cells, but were not significantly altered in mice received LicE. The weight of kidney was not affected by 4T1 cell injection or LicE administration (Supplementary Table S1). In addition, the levels of creatinine and the activities of AST and ALT in the sera were not detectably increased by either the injection of 4T1 cells or the administration of LicE (Supplementary Table S2).

Oral administration of LicE decreases cell proliferation and increases apoptosis in 4T1 mammary tumor tissues in BALB/c mice

IHC results revealed that the expression of Ki67 was lower in the tumor tissues of mice fed LicE as compared with control mice. In addition, the administration of LicE induced a significant reduction in the expression of CDK4, CDK2, cyclin A, and cyclin D1 in the tumor tissues (Fig. 2A and B).

A small number of TUNEL-positive apoptotic cells were detected in tumor tissues of mice received vehicle. The number of TUNEL-positive cells was increased in a LicE dose-dependent manner (Fig. 3A and B). In addition, the administration of LicE increased the expression of Bax, a proapoptotic member of the Bcl-2 family, in a dose-dependent manner. LicE at dose of 14 mg/kg reduced the expression of Bcl-2, an antiapoptotic member of the Bcl-2 family and increased the expression of cleaved caspase-3, the activated form of caspase-3 in tumor tissues (Fig. 3A and C).

Oral administration of LicE decreases the expression of angiogenesis-, lymphangiogenesis-, and inflammation-related proteins in 4T1 mammary tumor tissues in BALB/c mice

IHC and IF staining results revealed that the expressions of VEGF-A and CD31 were decreased significantly in tumor tissues of mice treated with LicE. VEGF-receptor (R)2 was reduced in mice treated with 14 mg/kg LicE. In addition, the oral administration of LicE decreased the expression of VEGF-C and the LYVE-1–positive vessels in tumor tissues. The expressions of CD45, cyclooxygenase-2 (COX-2), inducible nitric oxide synthase (iNOS) and hypoxia inducible factor-1α (HIF-1α) were decreased significantly in mice that orally received LicE (Fig. 4A and B). Results from in vitro studies revealed that LicE (5, 10, and 20 μmol/L) dose dependently inhibited the tube formation of HUVEC and SVEC endothelial cells (Supplementary Fig. S2).

Oral administration of LicE decreases the level of various proinflammatory cytokines and angiogenesis/metastasis-related proteins in the lung tissues of 4T1 tumor-bearing mice

In an effort to determine whether LicE feeding could decrease the levels of a variety of proinflammatory cytokines/chemokines and angiogenesis-related proteins in lung tissues, we conducted protein arrays using lung tissue lysates pooled from 15 mice. The results of array analyses
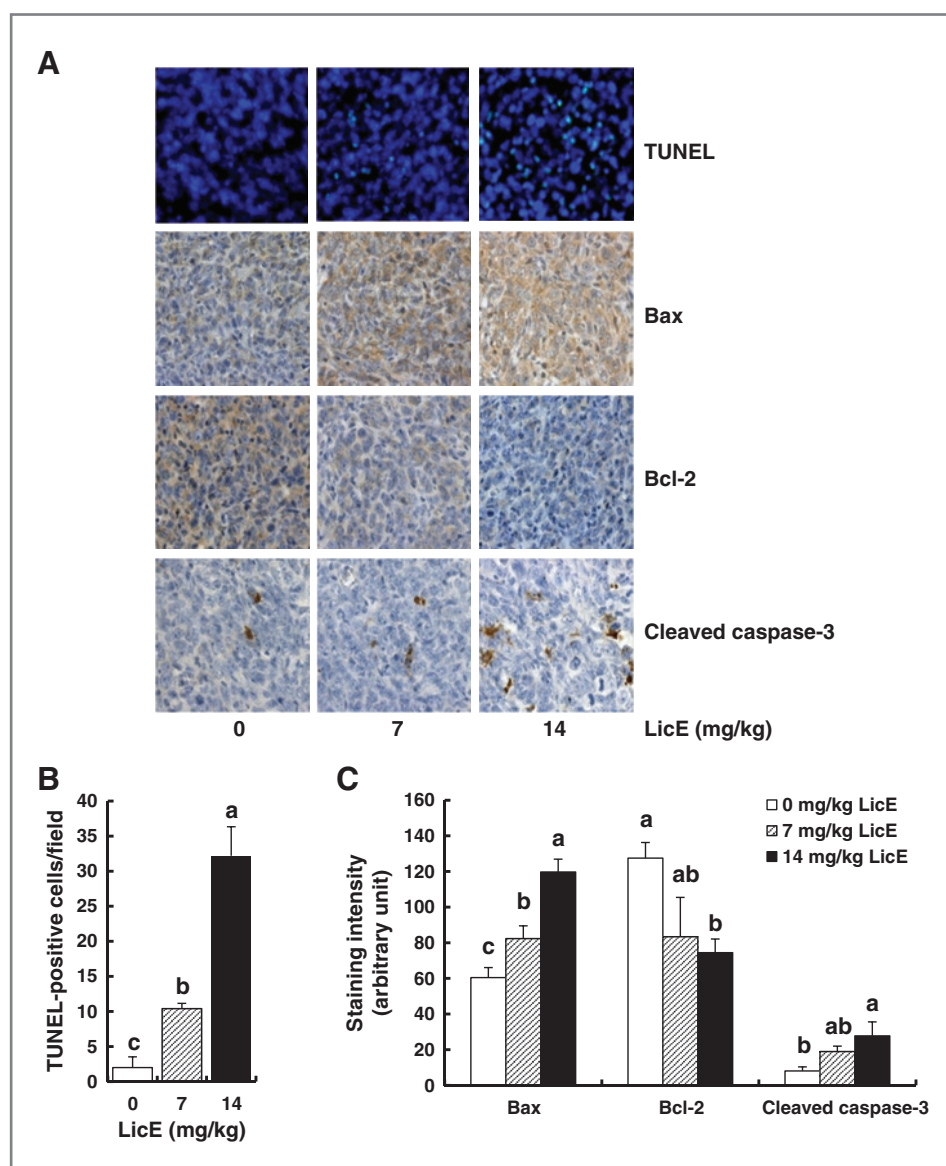
Figure 2. Oral administration of LicE decreases cell-cycle progression in 4T1 mammary tumors in BALB/c mice. Tumor sections were stained with an antibody raised against Ki67, CDK2, CDK4, cyclin A, or cyclin D1 and counterstained with hematoxylin. A, representative images of the IHC analysis are shown. B, Ki67, CDK2, CDK4, cyclin A, and cyclin D1-positive cells were counted. Each bar represents the mean ± SEM ($n = 5$). Means without a common letter differ, $P < 0.05$.
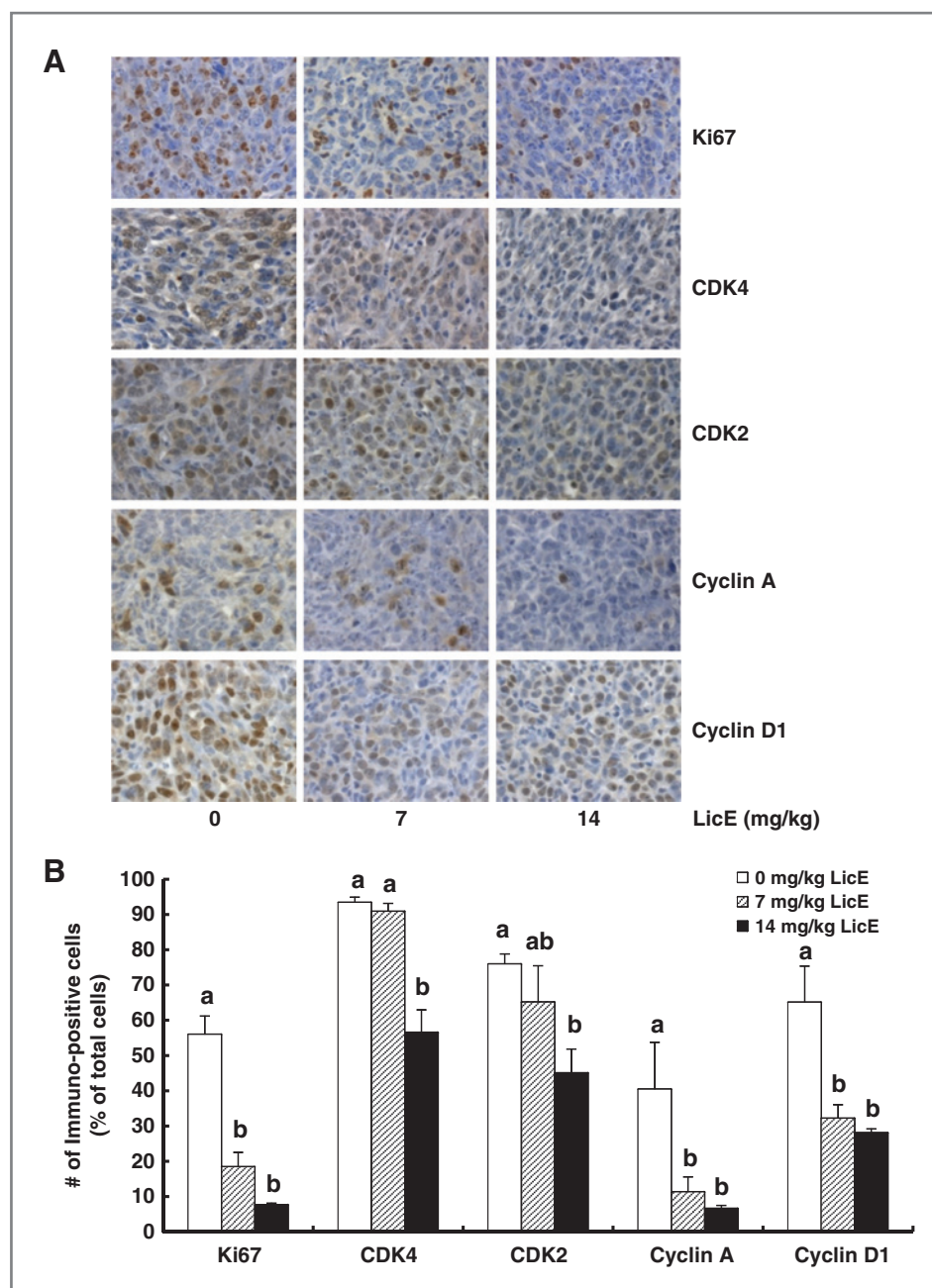
indicated that the levels of the following cytokines and proteins were markedly increased by 4T1 cell injection, which was noticeably decreased in mice fed LicE: complement component 5a (C5a), granulocyte colony-stimulating factor (G-CSF), IL-1ra, interferon gamma-induced protein 10 (IP-10; CXCL10), interferon-inducible T-cell–chemoattractant (I-TAC; CXCL11), keratinocyte-derived chemokine (KC), interleukin (IL)-1β, IL-16, macrophage-CSF (M-CSF), MCP-1, monokine induced by interferon-γ (MIG; CXCL9), regulated upon activation, normal T-cell expressed and secreted (RANTES; CCL5), TIMP-1, tumor necrosis factor-α (TNF-α), triggering receptor expressed on myeloid cells 1

(TREM-1), a disintegrin and MMP with thrombospondin motif-1 (ADAMST1), angiopoietin-3 (Ang-3), MMP-3, MMP-9, plasminogen activator inhibitor-1 (PAI-1), hepatopoietin A, and insulin-like growth factor binding protein-1 (IGFBP-1). In contrast, the levels of dipeptidyl peptidase IV (DPPIV; CD26), endoglin, endostatin, and chemokine (C-X-C motif) ligand 4 (CXCL4) were not noticeably increased by tumor cell injection but were reduced by LicE treatment (Supplementary Tables S3 and S4).

To examine the reliability of the difference in the levels detected by protein arrays, ELISAs with the individual lysate samples that had been used to pool for the array analysis

Figure 3. Oral administration of LicE increases apoptosis in 4T1 mammary tumors in BALB/c mice. Apoptotic cells were identified by TUNEL staining of tumor sections. Tumor sections were stained with an antibody raised against Bax, Bcl-2, or cleaved caspase-3 and counterstained with hematoxylin. A, representative images of TUNEL and the IHC staining are shown. B, the TUNEL-positive apoptotic cells were counted. C, the staining intensity of Bax, Bcl-2, and cleaved caspase-3 was quantified. Each bar represents the mean $\pm$ SEM ($n = 5$). Means without a common letter differ, $P < 0.05$.

were conducted. A strong correlation was observed between the protein levels determined by protein arrays and ELISAs. The ELISA results revealed that the levels of IL-1ra, MMP-9, and MCP-1 were increased in tumor cell-injected mice but decreased markedly in mice fed with LicE (Table 1).

LicE decreases the migration and invasion of breast cancer cells

As the oral administration of LicE markedly inhibited lung metastasis in mice, we determined whether LicE could directly inhibit the migration and invasion of mammary cancer cells. The results from the transwell migration and invasion assays showed that LicE (5, 10, and 20 µmol/L) dose dependently inhibited the migration and invasion of MDA-MB-231 (Fig. 5A and C) and 4T1 cells (Fig. 5B and D). The results of 3-(4,5-dimethyl-thiazol-2-yl)-2,5-diphenyl-

tetrazolium bromide (MTT) viability assays have shown that the same concentrations of LicE did not influence the viability of MDA-MB-231 (Supplementary Table S5) or 4T1 cells (Supplementary Table S6).

Since proteolytic cleavage of the extracellular matrix proteins is a necessary step in the migration of cancer cells, we next examined the effect of LicE on the secretion of TIMP-2, uPA, and MMPs in MDA-MB-231 cells. Results from Western blot analysis of 24 hour-conditioned media showed that LicE decreased the secretion of uPA and VEGF-A, whereas the secretion of TIMP-2 was increased (Fig. 5E, Supplementary Fig. S3A, S3B and S3C). Results from gelatin zymography revealed that LicE treatment decreased the secretion of pro MMP-9 but did not significantly affect the secretion of pro MMP-2 (Fig. 5F, Supplementary Fig. S3D).
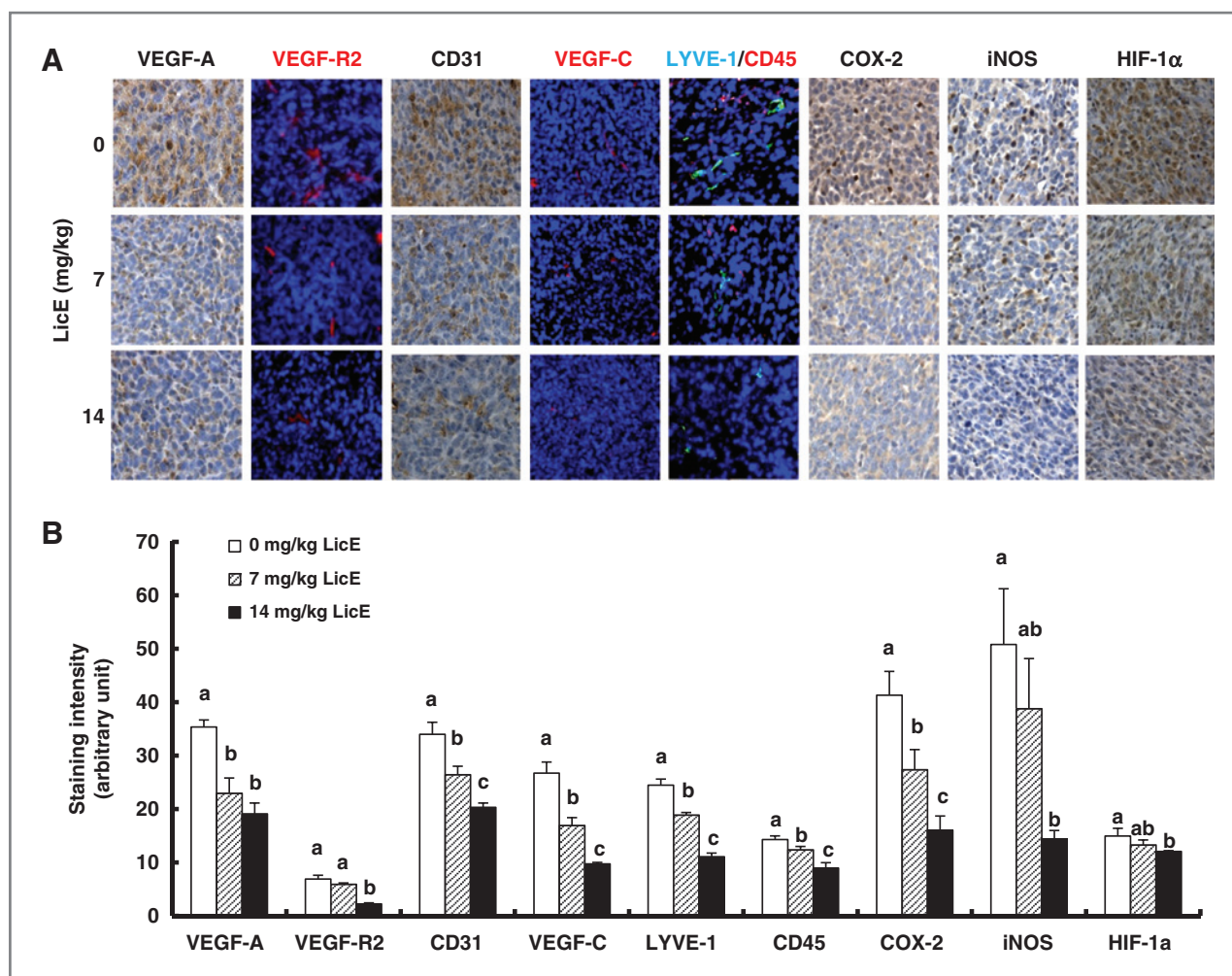
Figure 4. Oral administration of LicE decreases angiogenesis, lymphangiogenesis, and inflammation in 4T1 mammary tumors in BALB/c mice. Tumor sections were stained with an antibody raised against VEGF-A, VEGF-R2, CD31, VEGF-C, LYVE-1, CD45, COX-2, iNOS, or HIF-1α and counterstained with hematoxylin or 4′,6-diamidino-2-phenylindole (DAPI). A, representative images of the IHC or IF staining are shown. B, the staining intensity of VEGF-A, VEGF-R2, CD31, VEGF-C, LYVE-1, CD45, COX-2, iNOS, and HIF-1α were quantified. Each bar represents the mean ± SEM ($n = 5$). Means without a common letter differ, $P < 0.05$.

Discussion

In the present study, we show that LicE, a recently isolated retrochalcone present in licorice, exhibits potent anticancer effects in mice. We noted that LicE (i) inhibited solid tumor growth and lung metastasis in mammary tumor-bearing mice; (ii) inhibited tumor cell proliferation (Ki67+ cells) with reduced the expression of CDK2, CDK4, cyclin A, and D1 in tumor tissues;(iii) increased apoptosis (TUNEL+ cells) with increased expression of Bax and activated caspase-3 and decreased expression of Bcl-2 in tumor tissues; (iv) inhibited tumor angiogenesis (CD31+ cells) with decreased expression of VEGF-A and VEGF-R2; (v) inhibited tumor lymphangiogenesis (LYVE-1+ cells) with decreased expression of VEGF-C; (vi) inhibited the infiltration of inflammatory cells (CD45+ leukocytes) into tumor tissues with decreased expression of HIF-1α, iNOS, and COX-2; and(vii) reduced the levels of proinflammatory cytokines and proteins involved in the regulation of angiogenesis and metastasis in lung tissues. These effects were observed at the levels of LicE (7 or 14 mg/kg) that did not increase the serum levels of creatinine or activities of AST and ALT indicating that LicE exerts its anticancer effects at doses that do not cause toxicity to the kidney or liver. Recently, Park and colleagues reported that 2 weeks of LicE treatment (10 mg/kg) lowered blood glucose and triglyceride levels in high-fat diet-fed mice (10). However, we need to have much more information to relate the dose of LicE to a human treatment. To the best of our knowledge, no results have yet been published regarding LicE absorption and metabolism in animals. Future studies are needed to determine the concentrations of LicE and its metabolites in animal or human blood and tissues.

Oral administration of LicE resulted in reduced number of proliferating cells accompanied by reduced expression of CDK2, CDK4, cyclin A, and cyclin D1 (Fig. 2). In addition to decreased cell-cycle progression, apoptotic cell numbers

Table 1. Oral administration of licochalcone E (LicE) decreases the levels of IL-1ra, MMP-9, and MCP-1 in the lungs of 4T1 tumor-bearing BALB/c mice

	Sham injection	4T1 Cell injection		
LicE (mg/kg)	0	0	7	14
IL-1ra (ng/g)	13.8 ± 0.64	$34.0 \pm 1.81^{*a}$	27.9 ± 2.13^a	21.3 ± 2.49^b
MMP-9 (ng/g)	7.4 ± 0.3	$128.9 \pm 18.9^{*a}$	113.6 ± 23.3^a	38.3 ± 7.4^b
MCP-1 (pg/g)	63.4 ± 6.7	$316.6 \pm 52.5^{*a}$	224.7 ± 29.9^{ab}	160.1 ± 35.5^b

NOTE: Mice were injected with 4T1 cells (suspended in matrigel) or matrigel only (sham injection), and were fed by oral gavage with corn oil (vehicle) or LicE, and sacrificed as described in the Materials and Methods. LicE was diluted in corn oil, and all animals received the same amounts of corn oil. Four groups of mice were studied: (i) Sham-injected, 0 mg/kg LicE-fed, (ii) 4T1 cell-injected + 0 mg/kg LicE, (iii) 4T1 cell-injected + 7 mg/kg LicE, and (iv) 4T1 cell-injected + 14 mg/kg LicE. Lung tissue lysates were prepared for the determination of interleukin 1 receptor antagonist (IL-1ra), matrix MMP-9, and monocyte chemotactic protein-1 (MCP-1) levels by the respective ELISA kits. Values are expressed as the mean $\pm$ SEM ($n = 10$).
*Significantly different from the sham-injected group.
[a, b, ab]Means without a common letter differ between the 3 4T1 cell-injected groups. $P < 0.05$

were clearly increased in tumor tissues of LicE-treated mice (Fig. 3). In the present study, we did not investigate how the oral treatment of LicE reduced cycle progression and induced apoptosis in tumor tissues. However, it seems unlikely that LicE directly cause these changes. *In vitro* culture studies showed that LicE at 5 to 20 μmol/L inhibited the migration and invasion of 4T1 and MDA-MB-231 cells (Fig. 5A, B, C, and D) but did not cause any changes in cell viability (Supplementary Tables S5 and S6). The uncontrolled growth of solid tumor and metastasis are directly related to tumor angiogenesis by delivering oxygen and nutrients for the survival of rapidly proliferating tumor cells in addition to providing routes for the spread of tumor cells. Therefore, blocking the vascularization of incipient tumors can be a very effective strategy to inhibit tumor promotion (4). LicE treatment reduced CD31+ cells as well as the expression of VEGF-A and its mitogenic receptor VEGF-R2 in tumor tissues (Fig. 4). *In vitro* cell culture studies revealed that LicE directly inhibited the secretion of VEGF-A, a key factor in tumor angiogenesis (26) by MDA-MB-231 human breast cancer cells (Fig. 5E). These results suggest that LicE inhibited the secretion of VEGF-A by tumor cells, which resulted in inhibition of VEGF-R2 activation on endothelial cells leading to decreased tumor angiogenesis. In addition, *in vitro* studies revealed that LicE directly inhibited the tube formation of vascular endothelial cells (Supplementary Fig. S2). This reduction in tumor angiogenesis probably contributed to reduced solid tumor growth and lung metastasis in mice treated with LicE.

VEGF-C promotes tumor lymphangiogenesis and lymphatic metastasis (26, 27). Whether the metastasis to lymph nodes in human breast cancer takes place via preexisting lymphatic vessels or newly developed vessels has been a subject of debate (28–30). However, evidence showed that expression of VEGF-C in human breast cancer is strongly associated with lymphangiogenesis, lymphovascular invasion and lymphatic metastasis (31–33). In addition, it has

been shown that the induction of intratumoral lymphangiogenesis by VEGF-C overexpression in breast cancer cells promotes lung metastasis when human breast cancers were orthotopically transplanted onto nude mice (27). In our LicE-treated mice the expression of VEGF-C and LYVE-1, a specific marker for lymphatic endothelium (34), was significantly reduced in tumor tissues (Fig. 4). Together, these results suggest that LicE inhibits VEGF-C production in tumor cells, which inhibits tumor lymphangiogenesis leading to decreased lung metastasis in our mice.

HIF-1 activates the transcription of a wide variety of genes that are involved in multiple cellular functions including tumor growth, progression, angiogenesis, inflammation, and metabolism [reviewed in (35–37)]. The expressions of HIF-1α and its downstream target genes cyclin D, COX-2, iNOS, and VEGF-A were reduced in the tumor tissues of LicE-treated mice (Figs. 2, 4). Release of hypoxia-promoted inflammatory signals within tumor microenvironment recruits bone marrow-derived monocytes [reviewed in (38)]. In tumor tissues of LicE-treated mice, the infiltration of CD45+ leukocytes as well as the expression of COX-2 and iNOS, important mediators of inflammation (39), were significantly lower (Fig. 4). From these results, it can be postulated that the downregulation of HIF-1α by LicE might lead to reduced infiltration of leukocytes (CD45+ cells) in tumor tissues, contributing to the formation of tumor microenvironments that are less inflammatory and less favorable for the proliferation of tumor cells. Future studies are needed to determine the mechanisms by which LicE treatment inhibits HIF-1α activation.

In vitro cell culture results showed that LicE inhibited the migration and invasion of mammary cancer cells as well as the secretion of MMP-9 and uPA and increased the secretion of TIMP-2 (Fig. 5). These results indicate that LicE directly inhibits tumor cell migration and invasion via the inhibition of proteolytic activity in tumor cells. In addition to tumor cell virulence, changes in local tissue environment

Figure 5. Licochalcone E decreases the migration and invasion of mammary cancer cells. Transwell migration assays and invasion assays were conducted with serum-deprived MDA-MB-231 cells (A, C) and 4T1 cells (B, D) as described in the Materials and Methods. The number of migrated cells (A, B) or invaded cells (C, D) was quantified and expressed as a percentage of the untreated control cells. Each bar represents the mean ± SEM from 3 independent experiments. Means without a common letter differ, $P < 0.05$. E and F, serum-starved MDA-MB-231 cells were incubated in DMEM/F12 with 0 to 20 µmol/L LicE for 24 hours. Twenty-four hour-conditioned media were collected and concentrated for Western blotting (E) and gelatin zymography (F). The volumes of media loaded onto the gel were adjusted for equivalent proteins. Photographs of chemiluminescent detection of the immunoblots (E) and Coomassie blue stained gel (F), which were representative of 3 independent experiments, are shown. Serum-free HT-1080 cell-conditioned media was loaded as a positive control.
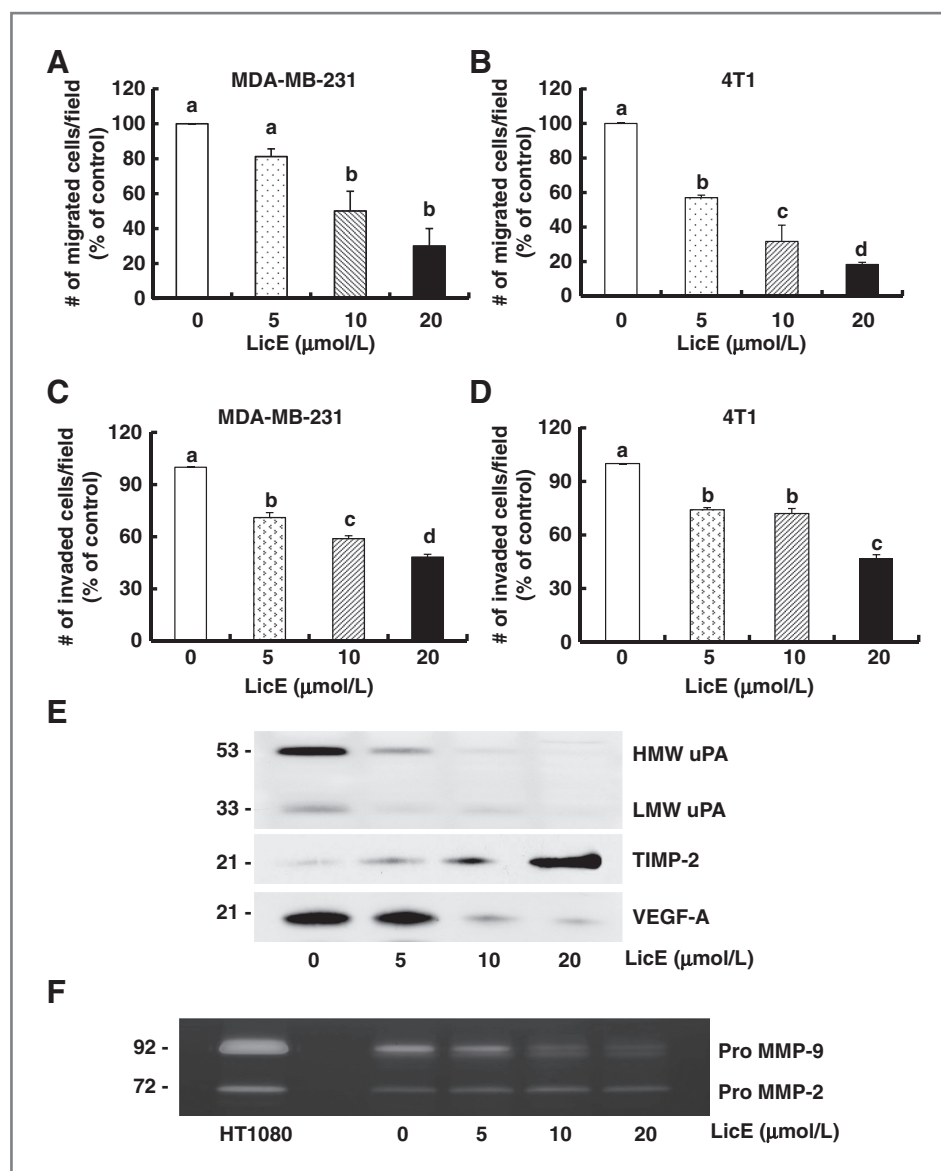
can be a critical component of tissue-specific metastasis. For example, the induction of MMP-9 by VEGF-R1 preceding tumor metastasis to the lung promotes lung metastasis (40). We noted that the levels of many proinflammatory cytokines and angiogenesis/metastasis-relate proteins were increased in the lung tissues of mice injected with tumor cells, which were markedly reduced in LicE-treated mice (Table 1, Supplementary Tables S3 and S4). Together, these results suggest that LicE induce changes in both tumor cells and lung microenvironment: these tumor cells become less aggressive and the lung microenvironment is less favorable for tumor cells to invade and proliferate.

In conclusion, LicE moderately inhibited solid tumor growth and potently inhibited lung metastasis in BALB/c mice orthotopically injected with syngeneic 4T1 mam-

mary carcinoma cells. These effects were accompanied by increased apoptosis of tumor cells and reduced tumor cell proliferation, migration, and invasion. LicE also induced a reduction in tumor angiogenesis and lymphangiogenesis as well as changes in local environment of tumor and lung tissues. The infiltration of leukocytes into tumor tissues, the expression of HIF-1α, as well as the proinflammatory enzymes COX-2 and iNOS in tumor tissues and levels of many proinflammatory cytokines in the lung were reduced in LicE-treated mice indicating that LicE exerts antiinflammatory effects in these mice. Since healthy people can harbor microscopic tumors in an asymptomatic and undetectable state for many years and these lesions do not progress in the absence of inflammation or angiogenesis (4), bioactive compounds such as LicE that can inhibit both tumor angiogenesis and

inflammation would be a very good potential candidate to prevent tumor progression.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

Authors' Contributions

Conception and design: K.W. Lee, M.-S. Choi, J.W. Yun, J.H.Y. Park
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